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Role of hypothalamic neuropeptide Y in a genetic model of obesity : the Zucker rat

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ROLE OF HYPOTHALAMIC NEUROPEPTIDE Y IN A GENETIC MODEL OF OBESITY - THE ZUCKER RAT

A Thesis Submitted to the Yale University School of Medicine in Partial Fulfillment of the Requirements for the Degree of Doctor of Medicine

by

Katherine E. Martin

1994

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ROLE OF HYPOTHALAMIC NEUROPEPTIDE Y IN A GENETIC MODEL OF OBESITY - THE ZUCKER RAT. Katherine E. Martin and Streamson C. Chua, Jr. Rockefeller University, New York, NY. (Sponsored by John N. Forrest, Jr., Department of Medicine, Yale University School of Medicine)

Human obesity is considered to have both behavioral and genetic components. The Zucker rat provides a valuable model of an inherited obesity syndrome caused by an unidentified autosomal recessive mutation of the *fatty (fa)* gene on chromosome 5. The development of obesity in animal models appears to be influenced by hypothalamic neuropeptide Y (NPY), via metabolic aberrations inducing hyperphagia, hyperinsulinemia, and energy conservation mechanisms. Elevated hypothalamic NPY levels have been demonstrated in obese adult Zucker rats. However, until recently, it has not been possible to assess the causal role of NPY in the development of this obesity, because the genotype of Zucker rat pups could not be determined prior to the onset of visible obesity. The creation of a new rat cross (13MxBN), similar to the Zucker rat strain (13M/13M), and identification of polymorphic genetic markers flanking the *fa* locus has permitted genotyping of 13MxBN rats at any age. The purpose of the present study was to elucidate the potential role of hypothalamic NPY in the development of the obese phenotype of the *fatty* rat, by comparing hypothalamic NPY levels in lean and preobese 13MxBN rat pups, using the

RT-PCR and Northern blot methods. The RT-PCR technique, which requires amplification of target sequence, showed no statistically significant difference between 6-8 day-old lean and preobese pups. However, the Northern blot method, which detects native mRNA, demonstrated a significant elevation of ~40%, ($p<.021$), in preobese rat pups at 7-8 days of age, compared to their lean littermates. Furthermore, the Northern blot results are consistent with those recently obtained with an RNAase protection assay. These studies provide evidence that elevated hypothalamic NPY is one of the earliest known molecular abnormalities in a genetic animal model of obesity, and suggest that NPY is integrally involved in producing the obese phenotype.

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I would like to thank my Rockefeller University advisors Streamson C. Chua, Jr., M.D./Ph.D., and Rudolph L. Leibel, M.D. for all their advice and assistance in completing this research. Further, I would like to thank John N. Forrest, Jr., M.D., for being my Yale thesis advisor, and also for his advice and assistance in completing this presentation of my work.

Introduction

Genetics of Human Obesity:

Human obesity is a common familial health disorder of developed countries that is associated with hypertension, non-insulin dependent diabetes mellitus (NIDDM), cardiovascular disease, and diminished longevity.[42] Until recently, obesity was primarily attributed to gluttony, but accumulating evidence suggests that obesity is determined by genetic factors.[6,8,21,41,42,53,54] Twin studies of body weight have shown that concordance rates for obesity in monozygotic twins are twice as high as those of dizygotic twins,[53] suggesting that genetic similarity plays a much stronger role in the development of obesity than familial environment. However, since most pairs of twins were raised in the same household, these studies were unable to sufficiently distinguish between the separate contributions of hereditary and environmental influences. Thus, adoption studies were performed which demonstrate that the weight of adult adoptees is far more closely correlated with that of their biological parents, than that of their adoptive parents. This finding further implicates genetic factors in the determination of an individual's weight.[41,54] Another observation favoring the hereditary hypothesis is the striking prevalence of obesity in certain genetically homogenous and geographically isolated populations. The Native American Pima tribe of Arizona comprises one such population, in which more than 75% of its members are obese, and, interestingly, more than 45% have

NIDDM.[42] The Pima tribe provides an unusual human model of genetic obesity and diabetes, which has been extensively studied.[6,42] In addition, there are rare cases of human obesity that follow a clear pattern of Mendelian inheritance,[21] and there are several genetic animal models of obesity thought to be the result of a single autosomal recessive mutation.[11,21] The availability, for research purposes, of multiple genetically obese animal models further facilitates our understanding of the complex pathogenicity of human obesity.

Metabolism of Obesity:

Obesity is a disorder of fuel metabolism that cannot be exclusively attributed to hyperphagia.[36,28,43,44] Although extremely prevalent, obesity is not well understood because it is likely to be a multifactorial disease.[28,40,42,44] Potential sites of deranged energy metabolism in the obese include adipose tissue, muscle tissue, and the hypothalamus.[28,36,37] Of these, the hypothalamus is the highest center involved in the control of eating behavior,[28] and it is postulated that the hypothalamus actually determines a body weight "set point", such that any perturbations in weight will induce mechanisms designed to return the body to its original weight.[33,40] Two hypothalamic neurotransmitters integrally involved in food intake and body metabolism include corticotropin releasing factor (CRF),[11a] and neuropeptide Y (NPY).[17,46,49-52] CRF inhibits feeding,[11a]

while NPY is the most potent orexigenic (appetite stimulating) agent known.[51] NPY is also known to stimulate release of the anabolic hormone insulin,[39] and one of the primary endocrine derangements associated with obesity is insulin resistance.[11a,42] Many aspects of energy metabolism in obesity need further clarification, and one strategy is to investigate the neuroendocrine control of this function in a genetic model of obesity.

Zucker Rat Model of Obesity and Exploration of Mechanisms:

The obese Zucker rat provides a unique genetic model for the study of the development of obesity. This obese phenotype is produced by an autosomal recessive mutation of the *fatty* (*fa*) locus, which arose spontaneously in the Zucker (13M) rat strain.[59] Although the specific gene has not yet been identified, the locus has been mapped to rat chromosome 5, and is homologous to the mouse *diabetes* (*db*) mutation on chromosome 4.[56] The obese Zucker syndrome is characterized by hyperphagia, hyperinsulinemia, insulin resistance, altered metabolism, hypothyroidism, and defective reproductive function.[10] However, the exact mechanism(s) triggering the obese *fatty* phenotype have not yet been delineated.

The Zucker obesity syndrome is characterized by the early onset of vastly increased fat storage, both in terms of adipocyte number and size.[10] Direct measurements reveal increased deposition of white adipose tissue by the fifth day

of life,[31] and the *fa/fa* rats are visibly obese by day 16, prior to the onset of hyperphagia at three weeks of age. Since hyperphagia does not manifest itself until after the obesity is evident, it clearly cannot be the causative factor of the obesity. In addition, it has been shown that *fa/fa* rats become obese even when their food intake is limited to, or less than that of their lean littermates,[10] suggesting that the obese animals have an increased efficiency of fat storage.[19] Of further note, the physical activity of lean and obese rats is similar, thus the weight gain of *fa/fa* rats cannot be attributed to reduced energy expenditure on activity.[19]

A prominent phenotypic feature of the *fatty* rat, promoting obesity, is altered metabolism.[10] By the second day of life, defective non-shivering thermogenesis is apparent in *fa/fa* rat pups, which impairs the ability of these animals to generate heat. Fuel that would otherwise be utilized in this metabolic process is deposited as adipose tissue, and is probably the most important source of energy for adipocyte storage in young *fa/fa* rat pups. However, when *fa/fa* pups are artificially reared slightly above the thermoneutral zone to circumvent their thermogenic defect, the *fa/fa* pups still deposit more fat than their lean littermates. Thus, defective thermoregulatory thermogenesis cannot be the primary cause of obesity in the Zucker rats.[31] In another study, sympathetic stimulation of metabolism, with norepinephrine (NE), in *fa/fa* and lean rat pups was shown to

arrest the onset of obesity in the *fa/fa* pups until 16 days of age. After this age, despite continued NE administration, the preobese pups accumulated excess fat which could not be entirely accounted for by a decreased metabolic rate. This study demonstrates that defects in sympathetically controlled metabolism are not solely responsible for the development of obesity.[35]

Any investigation into the triggering mechanism(s) of obesity in the Zucker *fa/fa* rat must focus on the peri-natal period because of the early onset of the obese phenotype, by day 5. This assumption precludes logical exploration of later developmental changes as precipitating causes of the obesity. For example, the alterations associated with the transition from suckling to independent, adult-like eating of solid food takes place about the twentieth day of age. Further, the transformation in the hormonal and metabolic milieu seen at puberty does not begin to take place until around day 60. None of the physiological changes associated with these later developmental milestones can be causally responsible for the obesity. To discover the cause, an appropriate area of investigation is the neuroendocrine regulation of eating behavior in *fa/fa* Zucker pups during the first week of life.

Role of Neuropeptide Y in Obesity:

Neuropeptide Y (NPY), a 36 amino acid member of the pancreatic polypeptide family, was discovered to be a

neurotransmitter that dramatically stimulates feeding behavior.[17] NPY is synthesized in the hypothalamic arcuate nucleus and the noradrenergic neurons of the brainstem which project to the hypothalamus. Injection of NPY intracerebroventricularly or directly into either the paraventricular nucleus (PVN) or ventromedial nucleus (VMN) of the hypothalamus stimulates feeding behavior, and decreases latency to feeding.[17,51,52] Long-term intracerebroventricular (ICV) administration of NPY produces hyperphagia and eventually obesity.[50] Furthermore, central administration of NPY triggers energy conservation mechanisms which decrease core temperature,[30] lower heart and respiratory rates,[26] and stimulate insulin release to promote energy storage.[39] Food deprivation produces elevated hypothalamic NPY levels in mice,[15] and rats.[4,9,29,46,48] There is, however, no change in brainstem levels of NPY with fasting,[15] which indicates that alterations in NPY synthesis are site specific. These observations all suggest that hypothalamic NPY levels are integrally involved in the control of eating behavior.

There is evidence that NPY plays a central role in the obesity syndrome of the *fa/fa* Zucker rat.[3,29,48] It has been consistently demonstrated that obese adult Zucker rats have elevated hypothalamic NPY levels compared to their lean counterparts. Both NPY messenger ribonucleic acid (mRNA) and NPY peptide levels have been shown to be increased in adult *fa/fa* rats of various ages.[3,29,48] Given that NPY is known to

stimulate eating behavior[17,51,52] and energy conservation mechanisms,[26,30,39] and that it is elevated in adult obese Zucker rats, it is likely that NPY is involved in the pathogenesis of obesity. The *fatty* gene, however, is not thought to be a mutation of the NPY locus, since the two genes are located on separate chromosomes. Furthermore, it is not clear if NPY is important in the early developmental expression of the obese phenotype in *fa/fa* rat pups. This research aims to resolve this intriguing question by comparing hypothalamic NPY levels in preobese and lean Zucker rat pups prior to the visible onset of obesity.

Genotype Determination of Rat Pups:

Until recently, it was difficult to determine the role of NPY in producing obesity in the *fa/fa* rat, because of the inability to easily and reliably distinguish preobese from lean pups prior to the visible expression of the obese phenotype. Several developments occurred which now allow early genotyping of rat pups. The obese Zucker phenotype which arose on the 13M strain background[59] was introduced into a genetic cross between two strains: 13M and Brown Norway (BN), with *fa* co-segregating with the 13M allele. Mapping of the *fa* mutation,[56] to rat chromosome 5, allowed the identification of two microsatellites flanking the *fa* locus, *Glut-1* and *c-Jun*,[14] thereby permitting the creation of assays to genotype rats at a very early age.

Determination of NPY mRNA:

For these studies it is preferable to detect NPY mRNA, rather than peptide levels, because mRNA is synthesized and stored in the neuronal cell bodies where it is produced. NPY peptides, however, are transported to synaptic vesicles which may be located in completely different areas than their corresponding cell bodies. In the hypothalamus, peptide levels partially represent synthesis in other areas, most notably the brainstem.[15] Thus, NPY mRNA levels were assessed using both the traditional Northern Blot technique, described in detail elsewhere,[15] and the newer Reverse Transcriptase - Polymerase Chain Reaction (RT-PCR) method.

RT-PCR is based on the polymerase chain reaction, which is an extremely sensitive method for detecting a known deoxyribonucleic acid (DNA) sequence which may be present in very low copy number. There are three distinct stages of the polymerase chain reaction which occur at corresponding temperature plateaus: denaturation of complementary DNA template strands, annealing of primers to template, and synthesis of the opposing DNA strands. Two oligonucleotide primers are designed to anneal to the 5' ends of opposite strands of the template DNA sequence. A heat stable DNA polymerase, from *Thermus aquaticus* (Taq), is then used to synthesize the complementary DNA sequence between the two primers. In order to detect the presence of the DNA product, the entire reaction must be repeated many times (ie. usually 20-35 times). In the first several cycles the desired DNA

product accumulates exponentially to a concentration of 10^{-8}M, then linearly, and finally reaches a concentration of approximately 10^{-7}M at which little additional product is generated.[5] The polymerase chain reaction is thus a method which greatly amplifies the amount of a specific DNA sequence relative to all other nucleic acid sequences in the original reaction mixture.

Several modifications of the standard PCR must be made in order to quantitate NPY mRNA. Hypothalamic mRNA must first be reverse transcribed into complementary DNA (cDNA) because Taq DNA polymerase cannot use RNA as a template. Also the number of PCR cycles must be designed such that the product is still accumulating in an exponential manner; if the PCR continues cycling until the product concentration reaches the plateau region then any difference in original template cDNA amounts will be masked. In addition, to account for variations in the total amount of cDNA placed into the PCR, an actin product is used as an internal control. Since hypothalamic and brain stem levels of actin mRNA are known not to vary with fasting manipulations, actin should represent a constant fraction of total hypothalamic mRNA.[15] Thus, within a given hypothalamic sample the NPY level is normalized to the corresponding actin level, permitting comparison of the relative amounts of NPY in different samples. As with many other techniques, the RT-PCR method results in a relative quantification of NPY mRNA, rather than an absolute measure.

Materials and Methods

Animals:

All Zucker (13M/13M) rats were bred in a colony maintained at Vassar College. The F1 (*fa/+*) adult animals were derived from matings between obese (*fa/fa*) 13M males and lean (+/+) Brown-Norway (BN) females. The rat pups used in these experiments were derived from matings of F1 animals. All F1 and F2 rats were housed in the Lab Animal Resource Center (LARC) at Rockefeller University in plastic cages with corn cob bedding and kept on a 12 hour light/12 hour dark cycle with lights on at 06:30 Eastern Standard Time. All subjects were maintained on Purina rat chow and water ad libitum except as noted in text.

Rats were placed in a carbon dioxide-enriched atmosphere prior to decapitation, and organs were harvested immediately thereafter. The hypothalami were dissected as described by Glowinski and Iversen. Tissues were either immediately homogenized for RNA isolation, or frozen in liquid nitrogen and stored at -70°C.

RNA Purification:

Hypothalamic tissue was homogenized in 1 milliliter (ml) of GEM [7M guanidine hydrochloride(HCl), 25 millimolar (mM) EDTA, and 1% 2-mercaptoethanol (2-ME)]. The homogenate was extracted with 1/2 volume of a phenol and chloroform mixture (1:1), and the aqueous supernatant carefully aspirated. The

RNA was precipitated by the addition of 1/20 volume of 3M sodium acetate (pH 4.5), and 1/2 volume of ethanol. After freezing, the solution was thawed and the precipitated RNA was further purified by washing with 0.1 ml of 3M sodium acetate and 1% 2-ME, and then ethanol. The RNA pellet was dissolved in water with 1% 2-ME. Total RNA yield was quantified from the optical density (OD) at 260 nanometers (nm), assuming 1 OD=33 micrograms (mcg)/ml of RNA.

RT-PCR:

1 mcg of RNA was reverse transcribed in 20 mcl of 0.05 M Tris-HCl (pH 8.3), 0.075 M potassium chloride (KCl), 3 mM magnesium chloride ($MgCl_2$), 0.5 mM deoxynucleoside triphosphates (dNTP's), 10 mM Dithiothreitol (DTT), 100 ng oligo$(dT)_{18}$, and 200 units of M-MLV-reverse transcriptase. The reverse transcription occurred at 37° C for 30-60 mins. The reaction mixture was then diluted with 80 mcl of water prior to heat-inactivation of the reverse transcriptase and dissociation of RNA/DNA hybrids at 100° C for 5 mins. The PCR was performed using the primers actin 1 and actin 3, yielding a 378 base pair (bp) product corresponding to codons 235 to 360 of rat beta-actin, and the NPY primers alpha-CPON and NPY 61, producing a 225 bp product correlating to codons -28 to 47 of rat NPY(See Table 1). Two mcl of cDNA were added to 20 mcl of 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 0.2 mM dNTP's, 2.5 mM $MgCl_2$, 0.2 mM spermidine, 250 ng/100 mcl each of the actin and NPY primers, 5 U/100 mcl

Taq polymerase, 0.25-0.5 mcl of ^{35}S-deoxyadenosine triphosphate (dATP), specific activity (1000 Ci/mmol and 10mcCi/mcl), and water up to a final volume of 22 mcl. To produce cleaner PCR products the reaction was "hot-started" by separating equal amounts of buffer, $MgCl_2$, dNTP's, and spermidine into two separate tubes. The DNA template, primers, and radioisotope were placed into one tube, and the Taq DNA polymerase into the other tube. The primer tube was layered with paraffin oil and heated to 94°C for one minute, and then the Taq mixture was added. Twenty PCR cycles were run at 94° C to denature, 65° C to anneal, and 72° C to extend for 30 seconds at each step in the Perkin Elmer thermal cycler, or for 60 seconds at each step in the MJ Research thermal cycler. The PCR products were size fractionated by electrophoresis on 5% polyacrylamide (PA) gels, and the dried gels were exposed to Kodak XAR film. Levels of mRNA were measured on the autoradiogram by densitometry using an LKB Ultroscan XL laser densitometer. For each sample, mRNA levels were expressed as the ratio of NPY/Actin to control for differences in loading, transfer, and RNA integrity.

Table 1: DNA sequences of oligonucleotide primers used in a PCR of an actin and NPY product

Primer name	DNA sequence
actin 1	5' CTG GAG AAG AGC TAT GAG CTG CCT
actin 3	5' CTC CTG CTT GCT GAT CCA CAT CTG
alpha-CPON (NPY)	5' TGA AAT CAG TGT CTC AGG GCT GGA
NPY 61	5' ATG CTA GGT AAC AAG CGA ATG GGG

The primers actin 1 and actin 3 yield a 378 bp product corresponding to codons 235 to 360 of rat beta-actin. The NPY primers alpha-CPON and NPY 61 produce a 225 bp product correlating to codons -28 to 47 of rat NPY.

Northern Blot:

Prior to electrophoresis, hypothalamic mRNA was denatured in an equal volume of RNA loading dye (50% formamide, 20% formaldehyde, 1% 2-ME, 1 mcg/ml of ethidium bromide (Et-Br), 40 mM MOPS (pH 7), 20 mM sodium acetate, 1 mM EDTA, 20% glycerol, and 0.01 % Bromophenol blue) by heating at 65° C for 10 minutes. Size fractionation was achieved by electrophoresis through 1.5% agarose gels in MAE (40mM MOPS, 20 mM sodium Acetate, 1 mM EDTA), containing 8% formaldehyde, 1 mcg/ml of Et-Br, and 0.1% 2-ME. The running buffer was MAE with 0.1 mcg/ml of Et-Br and 0.01% 2-ME. The RNA was transferred by capillary blotting to nitrocellulose in 20X SSC (3 M sodium chloride [NaCl], 0.3 M sodium

citrate). The RNA was fixed by baking at 65° C for 2 hours under vacuum.

Both NPY and actin radioactive probes were generated. The NPY plasmid pBL-NPY was digested with Eco RI to excise the insert.[27] The actin probe is a PCR product corresponding to codons 235 to 360 of rat beta-actin. Total rat brain mRNA was reverse transcribed into cDNA and the product was amplified using the primers actin 1 and actin 3, previously described in the RT-PCR section. Both the NPY insert and actin fragment were electrophoresed on 1% agarose gels and purified on diatoms.[7] The fragments were radiolabeled with ^{32}P-deoxycytosine triphosphate (dCTP) using the random primer labeling method.[20]

Blots were wet in water and pre-hybridized (50% formamide, 5X SSC, 5X Denhardt's, 0.1 mg/ml Herring sperm DNA, 0.1% SDS, and 5% polyethylene glycol [PEG]) at 37° C for 1 to 2 hours. Hybridization with either the NPY or actin probe was performed in pre-hybridization solution at 37° C overnight. The blots were washed three times in 2X SSC with 0.1% SDS at 65° C. The blots were exposed to Kodak XAR film at -70° C. Stripping of hybridized probes was done by washing the blots twice in 50% formamide with 0.1X SSC at 65° C. Levels of mRNA were measured by densitometry as described in the RT-PCR section and expressed as the ratio of NPY/Actin.

DNA Isolation:

Rat tail, toe clips, spleens and other tissues were digested overnight at 65° in at least 20 volumes of 250 mcg/ml proteinase K, 10 mM Tris, 10 mM EDTA, and 1% SDS. The tissue digest was extracted with 1/2 volume of a phenol and chloroform mixture (1:1) and the aqueous supernatant was carefully aspirated. The DNA was precipitated with 1/3 volume of 10 M ammonium acetate and 2/3 volume of isopropanol. The DNA pellet was washed with ethanol and dissolved in 20mM Tris and 0.5 mM EDTA, pH 8.

PCR Genotyping:

DNA genotyping was performed by a PCR using rat *rGlut-1* and *C-Jun* primers. *Glut-1* primers 1 and 2(See Table 2) were used in a PCR mixture as previously described in the RT-PCR section, except no radioactive isotope was used. 35 PCR cycles were run at 94° C to denature, 68° C to anneal, and 72° C to extend. The PCR products were electrophoresed on 8% PA gels and visualized under ultraviolet (UV) illumination after Et-Br staining. There is a product size difference of approximately 15-20 base pairs between BN and 13M, with the 13M allele being larger than the BN allele

For rat *C-Jun*, primers 1 and 2 were initially used which produce a ~140 bp product,[14] and later *rC-Jun* 5 and 6 were used which yield a smaller (~120 bp) and somewhat cleaner product. (See Table 2). There is a product size difference of 2 bases between BN and 13M, with the 13M allele being

larger than the BN allele. The *C-Jun* reaction was also performed according to the protocol in the RT-PCR section except that the ^{35}S-dATP concentration varied from 0.1-0.25 mcl/tube. 35 PCR cycles were run at 94, 60, and 72 degrees Celcius. The *rC-Jun* PCR products were analyzed either as single-stranded DNA (ssDNA) on denaturing PA gels, or as double-stranded DNA (dsDNA) on non-denaturing PA gels. Dried gels were exposed to Kodak XAR film. Each gel had DNA from animals of known genotype [(*fa/fa*), (*fa*/+), and (+/+)] which served as reference controls.

Table 2: DNA sequences of *Glut-1* and *C-Jun* oligonucleotide primers used in PCR genotyping of the *fa* locus

Primer name	DNA sequence
primer 1 (*Glut-1*)	5' GAA TGA AGC TAA GAA TTG ACC TTA GGT
primer 2 (*Glut-1*)	5' GTC CAT GCC TGT CCT TTA GTG CTC TTG
C-Jun 1	5' GGG GTG CGG AGC CAG CTT CA
C-Jun 2	5' CGG AGG GCT TGG GTG GGA G
C-Jun 5	5' GCT TGG GTG GGA GTA GAG GT
C-Jun 6	5' TTC AAT CGC CCA GAT CAT TCA

rC-Jun primers 1 and 2 produce a 141 bp product.[14] *rC-Jun* 5 and 6 were yield a smaller 121 bp and somewhat cleaner product. There is a product size difference of 2 bases between BN and 13M, with the 13M allele being larger than the BN allele.

Statistical Analysis:

Non-parametric statistical analysis was performed because the small sample sizes (ie. average litter size 10, with only 25% *fa/fa* animals) of these experiments precluded the assumption of a normal distribution of hypothalamic NPY levels. Therefore medians rather than means were used, and all tests of statistical significance were performed using either the Mann-Whitney U test or Wilcoxon Rank sums test for non-parametric data, unless otherwise specified.

Credits:

All procedures were performed by this author, except those subsequently specified. Zucker rats were bred in a colony maintained at Vassar College. F1 animals were bred under the supervision of Streamson Chua at Rockefeller University, and F2 animals were bred under the supervision of the author and Streamson Chua. Rats were housed and cared for by the LARC at Rockefeller University. Rats were sacrificed and organs dissected by the author, Streamson Chua, and David Markel at Rockefeller University. Oligonucleotide primers actin 1, actin 3, NPY alpha-CPON, NPY 61, *Glut-1* primers 1 and 2, and *C-Jun* primers 1,2,5 and 6 were designed by Streamson Chua, and made by the Protein Sequencing Facility at Rockefeller University. The Northern blot assay performed on the six-week-old Zucker rats was done by Streamson Chua. The RT-PCR assay performed on this group of animals was done by the author. mRNA gel fractionation for the Northern blot assay performed on the 7-8 day-old F2 BNx13M rat pups was done by the author and Streamson Chua. Loraine Keogh, at Rockefeller University, assisted the author in radiolabeling the NPY and actin probes for the Northern blot. Splenic DNA isolation from the group of 7-8 day old rat pups was performed by David Markel, as was the *C-Jun* genotyping for these animals. All other DNA isolation, *C-Jun*, and *Glut-1* genotyping was performed by the author. Statistical analysis using the Mann-Whitney U test was performed with the assistance of Rudolph Leibel at

Rockefeller University. The non-bar format graphs were generated at the Media Resources Center at Rockefeller University.

Results

Validation of RT-PCR Method:

Elevations in rodent NPY mRNA due to food deprivation have been demonstrated by previous investigators, using several different methods including: Northern blot,[29] RIA,[4,46] RNase protection assay,[48] and in situ hybridization.[9] This robust phenomenon should therefore be reproducible and detectable by any method used. Thus, to evaluate the accuracy of the relatively new RT-PCR method, it was compared to prior results obtained using a standard Northern blot method. Six-week-old male Zucker rats were separated into groups of 4 animals each according to genotype [(+/+) vs. (*fa/fa*)], and feeding status [*ad libitum* fed vs. 24-hour fast]. The rats were sacrificed, the 4 hypothalami of each group pooled, and total RNA was isolated according to the protocol discussed in the Materials and Methods section. A Northern blot of NPY only, (actin levels were not determined), was performed and analyzed visually, which showed a striking increase in the NPY signal in both fasted groups. (See Figure 1). Note that there is an increase in the NPY message size range ($\leq$ 100 bp) in the fasted groups, in addition to the increase in amount of NPY. This difference in NPY mRNA length may be secondary to variable

poly-adenylation of mRNA, which has been documented to occur in rat hypothalamic oxytocin and vasopressin mRNA when expression of the genes is stimulated by dehydration.[12,57]

Figure 1: Northern blot determination of hypothalamic NPY mRNA Levels in 6-Week-Old Adult Lean and Obese Zucker Rats: Elevation in Fasted and Obese Groups*

1 2 3 4 5 6

* Zucker rats were separated into groups of 4 animals each according to genotype [(+/+) vs. (*fa/fa*)], and feeding status {*ad libitum* fed vs. 24-hour fast vs. refed after a 24-hour fast]. The rats were sacrificed and the 4 hypothalami of each group pooled. Northern blot results are shown here. Lanes are shown in the following order from left to right: 1)lean fed group, 2)lean fasted group, 3)lean refed group, 4)obese fed group, 5)obese fasted group, and 6)obese refed group. Visual analysis shows a striking increase in NPY in the fasted groups. Note that there is an increase in the NPY message size range ($\leq$ 100 bp) in the fasted groups, in addition to the increase in amount of NPY. This difference in NPY mRNA length may be secondary to variable poly-adenylation of mRNA.

RT-PCR for both actin and NPY was performed on these same hypothalamic RNA samples according to the protocol in the Materials and Methods section. Triplicates were performed on each sample, which reveal a statistically

significant, ($p < .05$), ~120% elevation of NPY in the fasted groups when compared to the corresponding *ad libitum* fed groups. (See Table 3 and Figure 2). Note that there is also an approximate 70% increase in the NPY values of the obese as compared to the lean groups, which was significant in the fasted group, ($p < .05$), and bordered on statistical significance in the fed group, ($p = .05$). These RT-PCR values appear to correlate with the results obtained from the Northern blot, suggesting that the RT-PCR method is able to detect significant differences in NPY mRNA expression. Further, this experiment shows that the RT-PCR method can reproduce results which are expected from previous studies using other techniques.[2,3,47,48]

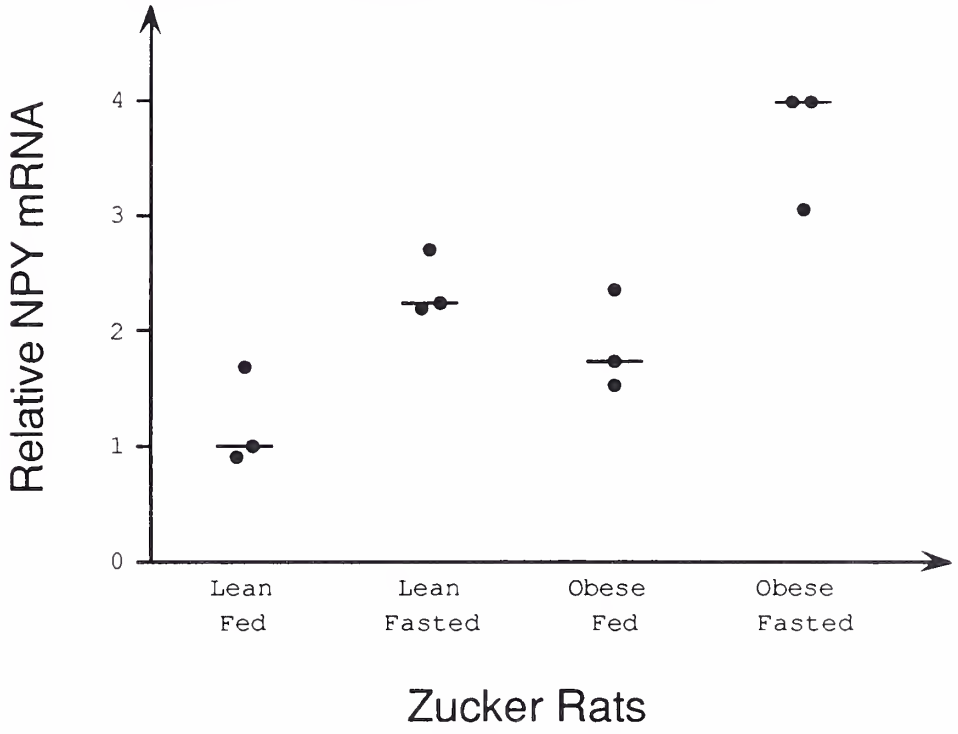
Table 3: Hypothalamic NPY mRNA levels of 6-week-old adult lean and obese Zucker rats: elevation in fasted and obese groups

animal	NPY/Actin mRNA* Triplicates			Median NPY/Actin mRNA
Lean - Fed	0.90	1.00	1.68	1.00
Lean - Fasted	2.20	2.24	2.71	2.24
Obese - Fed	1.52	1.74	2.36	1.74
Obese - Fasted	3.04	3.96	3.97	3.96

* NPY mRNA levels normalized to actin mRNA of the same sample. NPY/Actin levels normalized to median of the lean fed group.

Zucker rats were separated into groups of 4 animals each according to genotype [(+/+) vs. (*fa/fa*)], and feeding status [*ad libitum* fed vs. 24-hour fast]. The rats were sacrificed and the 4 hypothalami of each group pooled. Results reveal a statistically significant, ($p < .05$), ~120% elevation of NPY mRNA in the fasted groups when compared to the corresponding *ad libitum* fed groups. Note that there is also an approximate 70% increase of NPY in the obese as compared to the lean groups, which was significant in the fasted group, ($p < .05$), and bordered on statistical significance in the fed group, ($p = .05$).

Figure 2: Hypothalamic NPY mRNA Levels in 6-Week-Old Adult Lean and Obese Zucker Rats: Elevation in Fasted and Obese Groups*



* Zucker rats were separated into groups of 4 animals each according to genotype [(+/+) vs. (*fa/fa*)], and feeding status {*ad libitum* fed vs. 24-hour fast]. The rats were sacrificed and the 4 hypothalami of each group pooled. Triplicate RT-PCR assays were performed on each pooled sample. NPY mRNA levels were normalized to actin mRNA of the same sample. NPY/actin levels were expressed by normalization to the median of the lean fed group. Triplicates are shown with bars representing medians of the triplicates. Results reveal a statistically significant, ($p<.05$), ~120% elevation of NPY mRNA in the fasted groups when compared to the corresponding *ad libitum* fed groups. Note that there is also an approximate 70% increase of NPY in the obese as compared to the lean groups, which was significant in the fasted group, ($p<.05$), and bordered on statistical significance in the fed group, ($p=.05$).

Adult Obese 13MxBN F2 Rats Also Have Higher NPY Levels:

Several investigators, using various techniques, have shown increased hypothalamic NPY mRNA and peptide expression

in adult obese Zucker rats when compared to their lean counterparts.[3,29,47] However, no analysis of NPY levels has been performed on adult F2 rats from the 13MxBN cross which segregates the recessive obese phenotype with the 13M alleles. The RT-PCR method which was shown to detect NPY elevations in obese Zucker rats, was employed on a group of 11-week-old 13MxBN F2 rats to determine whether NPY levels are also increased in the obese animals of this cross. Three obese (*fa/fa*) and three lean controls were identified by gross phenotype, using the body mass index (BMI) which provides a measure of fatness which is independent of length. (See Table 4 and Figures 3, 4, and 5). PCR genotyping was not performed since genotyping is less accurate than phenotyping of adult animals.

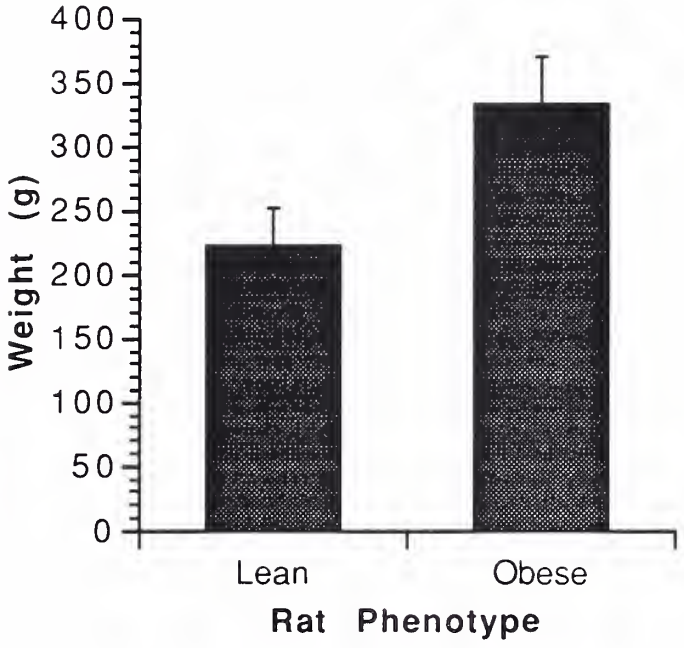
Table 4: Sex, weight, length, and BMI* of lean and obese 11-wk-old 13MxBN F2 Rats

Phenotype	Sex	Weight (g)	Length (cm)	BMI
Lean	F	177.2	19.2	.481
Lean	F	206.7	19.7	.533
Lean	M	281.1	21.5	.608
Obese	F	264.0	19.3	.709
Obese	M	336.1	21.8	.707
Obese	M	398.1	22.3	.801

$BMI = wt/(length)^2$

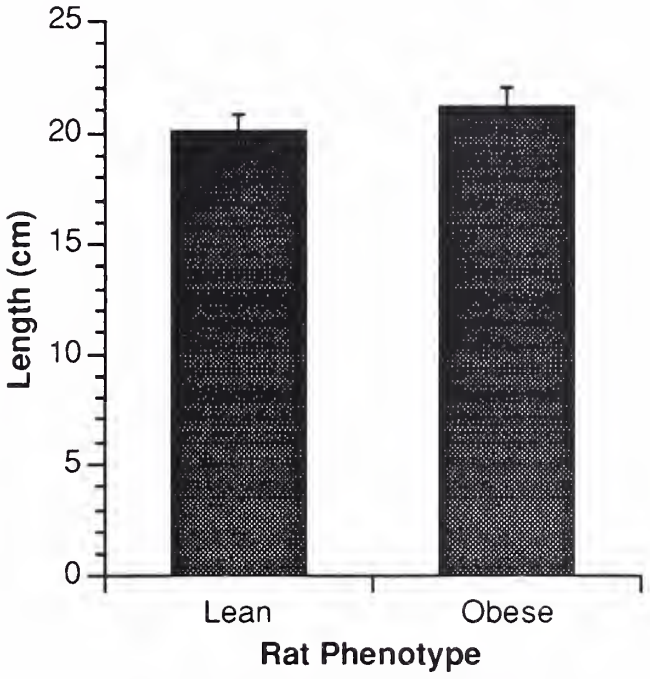
Statistical comparison between lean and obese animals reveals significant difference for weight, $p<.05$, and BMI, $p<.01$.

Figure 3: Weight of Lean and Obese 11-Week-Old Adult 13Mx BN Rats: Elevation in the Obese Group*



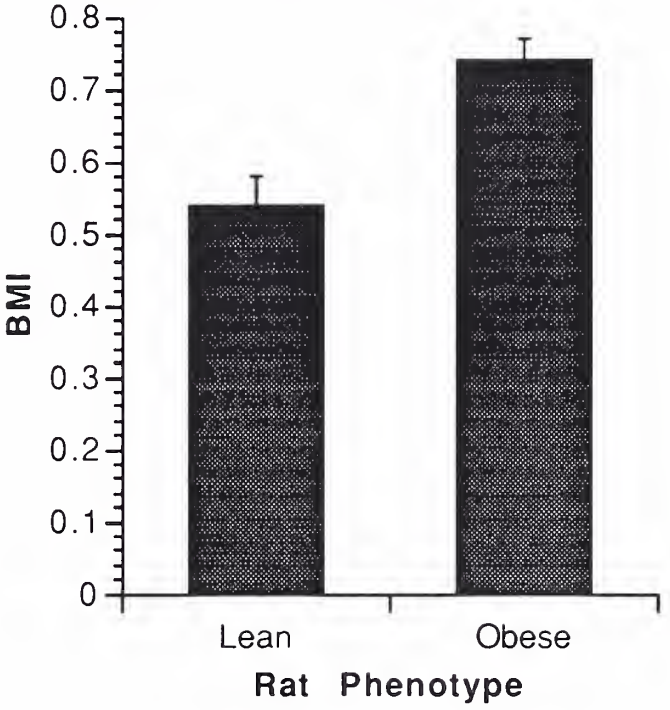
* Rats were separated into 2 groups by gross phenotype. Weight was measured to the nearest 0.1 gram. Means are shown, with standard error bars. The weight of the obese group is statistically significantly higher, one-tailed probability using the Student's Unpaired t-Test, (p=.0443).

Figure 4: Length of Lean and Obese 11-Week-Old Adult 13MxBN F2 Rats: No Difference by Phenotype*



* Rats were separated into 2 groups by gross phenotype. Length was measured to the nearest 0.1 cm. Means are shown with standard error bars. Results show no statistically significant difference between the 2 groups, ($p>.05$), using the Student's Unpaired t-Test.

Figure 5: BMI of Lean and Obese 11-Week-Old Adult 13MxBN F2 Rats: Elevation in the Obese Group*



* Rats were separated into 2 groups by gross phenotype. The BMI is equal to the weight divided by the length squared. Means are shown, with standard error bars. The weight of the obese group is statistically significantly higher, one-tailed probability using the Student's Unpaired t-Test, ($p<.01$).

Total hypothalamic mRNA was isolated, reverse transcribed, and the PCR performed using both NPY and actin primers as outlined in the Materials and Methods section. Triplicates were performed on each hypothalamic sample. The medians of the NPY/actin triplicates, 1.00 for the lean group and 1.51 for the obese group, show that there is a statistically significant, ($p < .05$), elevation in NPY expression of approximately 50% in the obese animals. (See

Table 5 and Figure 6). Note that a previous study of 11-week-old Zucker rats, using the Northern blot technique, did not demonstrate any difference between lean and obese animals,[29] whereas this experiment detected a significant, though not large, increase in the F2 obese group. This discrepancy might be accounted for by the two different genotypic background strains used. This experiment shows that the RT-PCR method is capable of detecting small elevations of NPY mRNA, similar to those found in obese Zucker rats, in adult obese 13MxBN F2 rats when compared to lean rats of the same age.

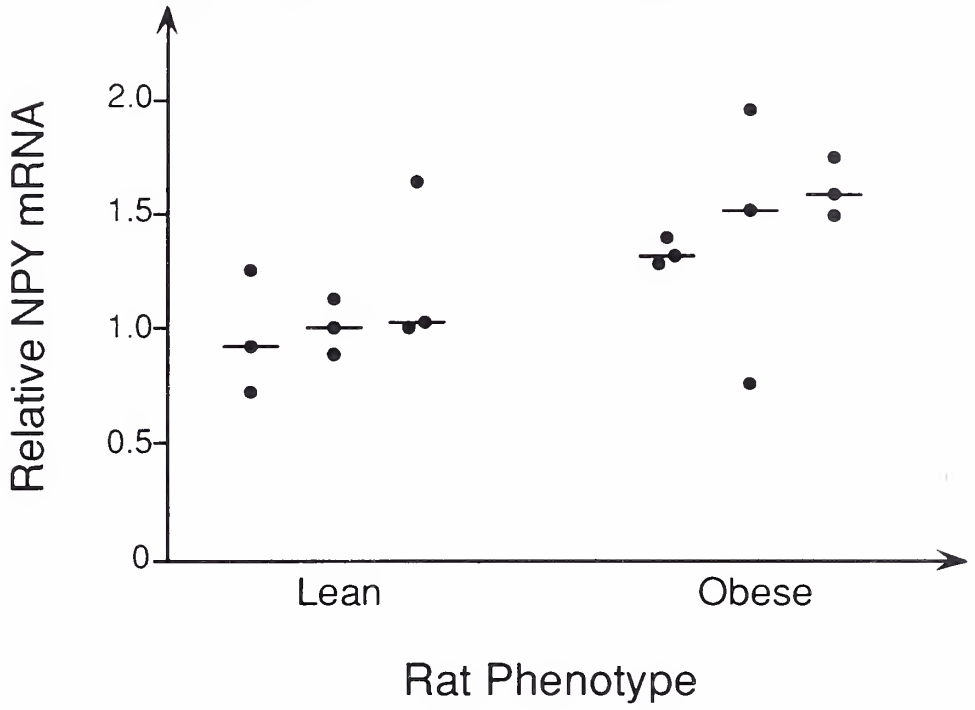
Table 5: Hypothalamic NPY mRNA levels of lean and obese 11-wk-old adult 13MxBN F2 rats: elevation in the obese group

Phenotype	Median & Range of Triplicate NPY/Actin mRNA Levels*	Median of Groups
Lean	0.92 (0.72-1.26)	1.00
Lean	1.00 (0.89-1.13)	
Lean	1.02 (1.00-1.64)	
Obese	1.31 (1.28-1.39)	1.51
Obese	1.51 (0.76-1.95)	
Obese	1.58 (1.49-1.74)	

* NPY mRNA levels normalized to actin mRNA of the same sample. NPY/Actin levels normalized to median of lean group.

Three obese (*fa/fa*) and three lean adult F2 13MxBN rats were identified by gross phenotype. The medians of the NPY/actin triplicates, 1.00 for the lean group and 1.51 for the obese group, show that there is a statistically significant, $p<.05$, elevation in NPY expression of approximately 50% in the obese animals.

Figure 6: Triplicate Hypothalamic NPY mRNA Levels in 11-Week-Old Adult Lean and Obese 13MxBN F2 Rats: Elevation in the Obese Group*



* 3 obese (*fa/fa*) and 3 lean adult F2 13MxBN rats were separated into 2 groups by gross phenotype. Triplicate hypothalamic NPY mRNA determinations were performed on each sample using the RT-PCR method. NPY mRNA levels were expressed by normalization to the median of the lean group. Triplicates are shown with bars representing medians of the triplicates. Results reveal a statistically significant, ($p<.05$), ~50% elevation of NPY mRNA in the obese group.

Rat Pup Genotyping Issues:

Rat pups were genotyped by two PCR based assays utilizing the rat chromosome 5 simple sequence repeat (SSR) polymorphisms for *Glut-1* and *C-Jun*. The distance of *Glut-1* from the *fa* locus is 8.5 centimorgans(cM), and the corresponding distance from *C-Jun* to *fa* is 4.5 cM. The results of the two assays were not always in accord, which is possible if recombination occurs between the markers. All

possible genotype combinations are shown in Table 6, and whether a particular combination was included in further statistical analysis. Pups that scored 13M//13M by only one assay were not used in the analysis since the phenotype at the *fa* locus could not be unambiguously determined. Note that a 13M/13M genotyping result in both assays is considerably more accurate than a single assay 13M/13M determination, since the microsatellites are located on opposite sides of the *fa* locus. Using both assays in conjunction results in a 1 in 66 chance of erroneously calling a *fa/fa* pup a lean animal, while using only *Glut-1* gives a 1 in 6 chance, and using *C-Jun* alone gives a 1 in 11 chance. A more detailed discussion of possible errors in assigning genotype at the *fatty* locus is given in Chua *et. al.*, 1993.[14]

Table 6: Possible genotype & phenotype results for F2 rat pups of the 13MxBN cross using rat chromosome 5 Single Sequence Repeat (SSR) polymorphisms for *Glut-1* and *C-Jun* in PCR linkage assays to the *fa* locus [t]

rGlut-1 Assay	*rC-Jun* Assay	Assigned Genotype	Phenotype	Statistical analysis*
13M/13M	13M/13M	*fa/fa*	obese	included
BN/13M	BN/13M	*fa*/+	lean	included
BN/BN	BN/BN	+/+	lean	included
BN/13M	BN/BN	*fa*/+ or +/+	lean	included
BN/BN	BN/13M	*fa*/+ or +/+	lean	included
13M/13M	BN/13M	unknown	?	discarded
13M/13M	BN/BN	unknown	?	discarded
BN/13M	13M/13M	unknown	?	discarded
BN/BN	13M/13M	unknown	?	discarded

[t] Rat pup genotyping is performed by PCR's in which 2 mcl of cDNA is added to 20 mcl of 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 0.2 mM dNTP's, 2.5 mM $MgCl_2$, 0.2 mM spermidine, 5 U/100 mcl Taq polymerase, 250 ng/100 mcl each of either the *Glut-1* or *C-Jun* primers, 0.1-0.25 mcl of ^{35}S-dATP for the C-Jun reaction only, and water up to a final volume of 22 mcl. 35 PCR cycles are run at temperature plateaus of 94, 68, and 72° C for *Glut-1*, and 94, 60, and 72° C for *C-Jun*. *Glut-1* PCR products are electrophoresed on 8% PA gels and visualized under UV illumination after Et-Br staining. *rC-Jun* PCR products are analyzed either as ssDNA on denaturing PA gels, or as dsDNA on non-denaturing PA gels. Dried gels are exposed to Kodak XAR film.

BN = + allele
13M = *fa* allele

* Rat pups which are 13M/13M by only one assay are not included in further statistical analysis.

Using both the *Glut-1* and *C-Jun* assays in conjunction results in a 1 in 66 chance of erroneously calling a *fa/fa* pup a lean animal.

RT-PCR Determination of Hypothalamic NPY Levels in 13MxBN F2 Rat Pups:

The RT-PCR assay was employed on young 13MxBN F2 rat pups to determine if hypothalamic NPY levels differ between preobese pups and their lean littermates. Three litters of rat pups were toe clipped on the second to third day of life for genotype analysis. The DNA was isolated from these toe clips and both the *rGlut-1* and *rC-Jun* PCR assays were performed on the DNA as outlined in the Materials and Methods section. Based on the results of the genotyping, 4 *fa/fa* and 9 [*fa*/+ or +/+] pups were selected for analysis of hypothalamic NPY expression. (See Table 7).

Table 7: Genotype and phenotype results for 6-day-old F2 rat pups of the 13MxBN cross using rat chromosome 5 SSR polymorphisms for *Glut-1* and *C-Jun* in PCR linkage assays to the *fa* locus*

rGlut-1 Assay	*rC-Jun* Assay	Assigned Genotype	# of Animals	Phenotype
13M/13M	13M/13M	*fa/fa*	4	Preobese
BN/13M	BN/13M	*fa*/+	4	Lean
BN/BN	BN/BN	+/+	5	Lean

BN = + allele
13M = *fa* allele

* RNA isolation of 7 of the original 24 rat pups was unsuccessful. Of the remaining 17 animals, 4 animals could not be unambiguously genotyped at the *fa* locus. Thus, 11 of 24 animals were excluded from this table, and from further statistical analysis.

The rat pups were sacrificed on the sixth day of life, and the hypothalami were harvested. Total hypothalamic mRNA was isolated, reverse transcribed, and assayed for NPY and actin mRNA by a PCR outlined in the Materials and Methods section. The results of the first assay, performed once on each sample, suggested only a minor increase in the NPY levels of the preobese rat pups, 1.09 vs. 1.00. The assay was repeated to further clarify these results, and triplicate measures were performed on each sample. (See Table 8).

The singlet and triplicate hypothalamic NPY/actin mRNA values were compared with one another, by normalizing each sample value to the control NPY/actin mRNA value obtained for the corresponding RT-PCR assay run. Inter-assay variability in efficiency of the RT and PCR steps occurs, and autoradiogram exposure time also differs. To account for such variability, hypothalamic measures must be expressed relative to a standardized control. Thus, during each run of the RT-PCR assay, the same whole rat brain RNA control was used. It is assumed that this control always has the same initial concentration of NPY and actin mRNA, and that the resulting NPY/actin mRNA level reflects assay efficiency and autoradiogram exposure time. Each sample was expressed as a fraction of the control value obtained for that particular assay run, thereby allowing inter-assay comparisons.

This group of rat pups had four separate measures of NPY and actin mRNA performed on each hypothalamic sample. The results indicate that the medians of the preobese and entire

lean group are identical (1.00 and 1.00) and that there is no statistically significant, ($p > .05$), difference between the lean and obese groups. The *fa*/+ group has a slightly higher mean of 1.20, but the values for this group are not statistically significantly different from the preobese group. However, the *fa*/+ group values are significantly higher than the +/+ group, (one-sided significance level $p = .032$). (See Table 8 and Figures 7 and 8). The comparison of singlet to triplicate measures shows that despite the variability in repeated measures on the same sample, a single determination of each sample is fairly reliable.

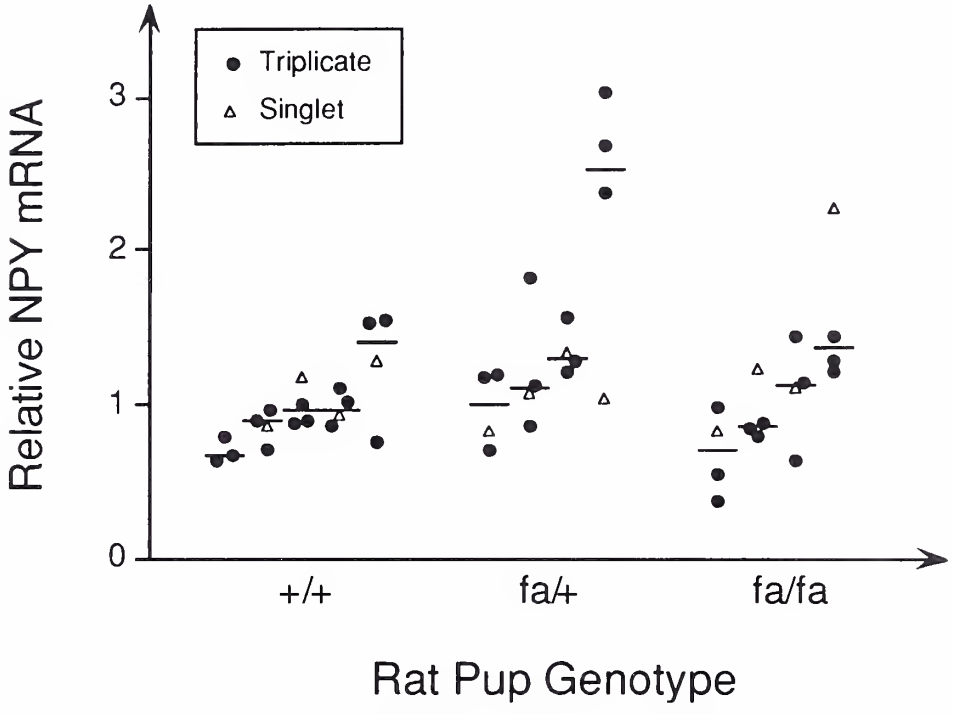
Table 8: Triplicate and singlet hypothalamic NPY mRNA* determinations of 6-day-old F2 rat pups of the 13M×BN cross suggest no difference between lean and preobese pups

Assigned Genotype	Triplicate NPY/Actin mRNA levels			Singlet NPY/Actin mRNA Level	Median	Median of groups
	1	2	3			
+/+	0.64	0.68	0.79	--	0.67	
+/+	0.71	0.90	0.96	0.87	0.90	
+/+	0.88	0.89	1.00	1.17	0.97	0.97
+/+	0.86	1.01	1.10	0.93	0.97	
+/+	0.76	1.52	1.54	1.27	1.40	
fa/+	0.71	1.18	1.19	.83	1.00	
fa/+	0.86	1.12	1.81	1.07	1.10	1.20
fa/+	1.20	1.28	1.56	1.33	1.30	
fa/+	2.37	2.68	3.03	1.03	2.53	
fa/*fa*	0.38	0.55	0.98	0.83	0.70	
fa/*fa*	0.80	0.84	0.88	1.23	0.87	1.00
fa/*fa*	0.63	1.14	1.43	1.10	1.13	
fa/*fa*	1.21	1.28	1.44	2.27	1.37	

* NPY mRNA levels normalized to actin mRNA of the same sample. All triplicate values are then normalized to the whole rat brain control NPY/actin mRNA value for that particular assay run, and all singlet values are normalized to the rat brain control value for that assay run. All values are then normalized to the median of the lean group.

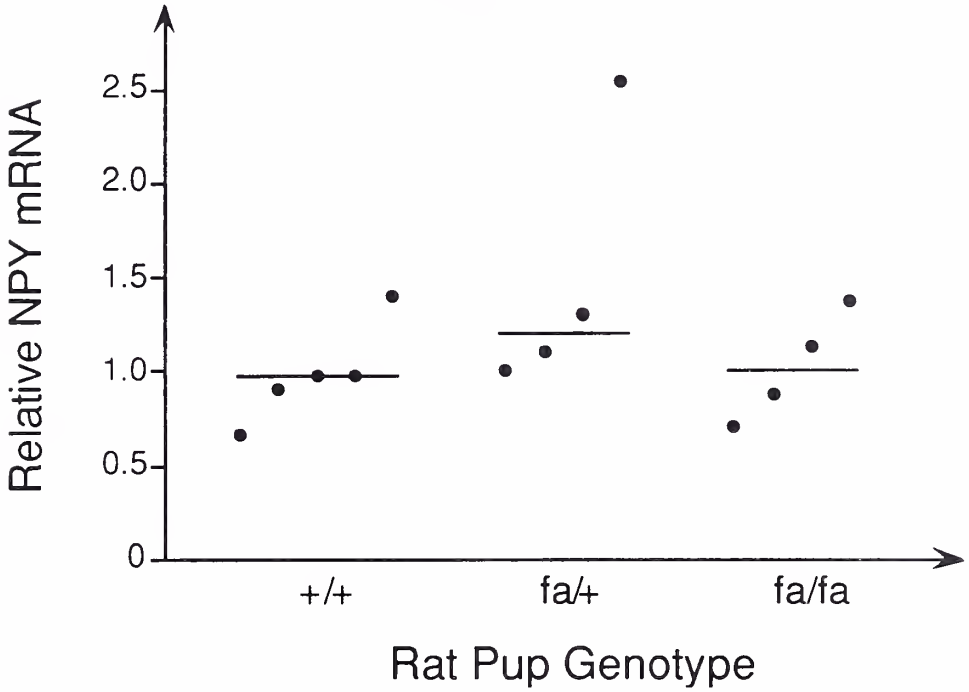
The results indicate that the medians of the preobese and entire lean group are identical (1.00 and 1.00) and that there is no statistically significant, $p>.05$, difference between the lean and obese groups. The *fa*/+ group has a slightly higher mean of 1.20, but the values for this group are not statistically significantly different from the preobese group. However, the *fa*/+ group values are significantly higher than the +/+ group, (one-sided significance level $p=.032$).

Figure 7: Triplicate and Singlet Hypothalamic NPY Levels in 6-Day-Old Lean and Preobese 13MxBN Rat Pups: No Difference Between Lean and Preobese Groups*



* Rat pups were genotyped using rat chromosome 5 SSR polymorphisms for *Glut-1* and *C-Jun* in PCR linkage assays to the *fa* locus. Triplicate and singlet hypothalamic NPY mRNA levels were determined using the RT-PCR technique. NPY mRNA levels were normalized to actin mRNA of the same sample. All triplicate values were then normalized to the whole rat brain control NPY/actin mRNA value for that particular assay run, and all singlet values were normalized to the rat brain control value for the singlet assay run. All values are expressed by normalization to the median of the entire lean group. Triplicate and singlet values are shown, bars represent medians. The results indicate that the medians of the *fa/fa* and entire lean group are identical (1.00 and 1.00) and that there is no statistically significant, ($p>.05$), difference between the lean and preobese groups. The *fa/+* group has a slightly higher mean of 1.20, but the values for this group are not significantly different from the preobese group, ($p>.05$). However, the *fa/+* group values are significantly higher than the +/+ group, (one-sided significance level $p=.032$).

Figure 8: RT-PCR Medians of Triplicate and Singlet Hypothalamic NPY Levels in 6-Day-Old Lean and Preobese 13MxBN Rat Pups: No Difference Between Lean and Preobese Groups*



* Rat pups were genotyped using rat chromosome 5 SSR polymorphisms for *Glut-1* and *C-Jun* in PCR linkage assays to the *fa* locus. Triplicate and singlet hypothalamic NPY mRNA levels were determined using the RT-PCR technique, medians are shown here. NPY mRNA levels were normalized to actin mRNA of the same sample. All triplicate values were then normalized to the whole rat brain control NPY/actin mRNA value for that particular assay run, and all singlet values were normalized to the rat brain control value for the singlet assay run. All values are expressed by normalization to the median of the entire lean group. Medians of the 4 measures for each animal are shown, with bars representing the medians of each group of animals. Medians of the preobese and entire lean group are identical (1.00 and 1.00) and there is no statistically significant, ($p>.05$), difference between the lean and preobese groups. The *fa*/+ group has a slightly higher mean of 1.20, but this is not significantly different from the *fa/fa* group, ($p>.05$). However, the *fa*/+ group values are significantly higher than the +/+ group, (one-sided significance level $p=.032$).

Comparison of RT-PCR and Northern Blot Methods:

The results of the RT-PCR method, which showed no difference between the NPY mRNA levels of preobese and lean 13MxBN F2 rat pups, conflict with that of a recent RNase protection assay study which detected a 50% elevation of NPY levels in 13MxBN rat pups of the same age.[16] To resolve these differing results, the RT-PCR and Northern blot methods were compared on a similar group of 13MxBN F2 rat pups. Three litters of rat pups (a total of 29 pups) were sacrificed on the 7th or 8th day of life. Spleens were harvested and DNA isolated for genotype analysis using the *rGlut-1* and *rC-Jun* assays. Two animals with ambiguous genotypes were discarded. The assigned genotypes at *fatty* in the remaining 27 animals approximated the expected Mendelian distribution of an autosomal recessive phenotype: 8 (30%) were preobese (*fa/fa*) and 19 (70%) were lean (*fa*/+ or +/+). (See Table 9).

Table 9: Genotype and phenotype determination of 7-8 day old F2 rat pups of the 13MxBN cross: approximates a Mendelian distribution

rGlut-1 Assay	*rC-Jun* Assay	Assigned Genotype*	# of Animals	Phenotype
13M/13M	13M/13M	*fa/fa*	8	Preobese
BN/13M	BN/13M	*fa*/+	10	Lean
BN/BN	BN/BN	+/+	5	Lean
BN/BN	BN/13M	*fa*/+ or +/+	2	Lean
BN/13M	BN/BN	*fa*/+ or +/+	2	Lean

BN = + allele
13M = *fa* allele

* Two animals that scored 13M/13M by only one assay were not included in this table or further analysis. The assigned genotypes at *fatty* in the remaining 27 animals approximated the expected Mendelian distribution of an autosomal recessive phenotype: 8 (30%) were preobese (*fa/fa*) and 19 (70%) were lean (*fa*/+ or +/+)

Rat pup hypothalami were harvested on the day of sacrifice for NPY mRNA analysis by the Northern blot method. Total hypothalamic mRNA was isolated and then electrophoresed through agarose gels and blotted with both NPY and actin probes as outlined in the Materials and Methods section. The results of the Northern blot show a statistically significant, ($p < .021$), increase in NPY mRNA of 37% in the preobese group (median 1.37) when compared to the lean group (median 1.00). Further statistical analysis suggests that this effect may only be significant when lean homozygote

(+/+) animals are considered in relation to their preobese counterparts. A comparison of the 5 +/+ animals to the 8 *fa/fa* animals shows a statistically significant elevation of NPY in the obese group, ($p < .040$), the median of the *fa/fa* group is 1.40 times that of the +/+ group. Comparison of 10 heterozygote (*fa*/+) lean animals to the 8 preobese (*fa/fa*) animals shows a median of 1.00 for the lean group and 1.30 for the preobese group, but this difference is not statistically significant ($p > .050$). (See Table 10 and Figure 9). Note that not all of the lean animals could be unambiguously classified as either +/+ or *fa*/+, thus not all of the animals could be included in this subset analysis. The exclusion of these 4 animals in the comparison between genotype classes may have decreased the power of the subset analysis.

Table 10: Northern blot determination of hypothalamic NPY mRNA levels for 7-8 day old lean and preobese F2 rat pups of the 13MxBN cross: elevation in the preobese group

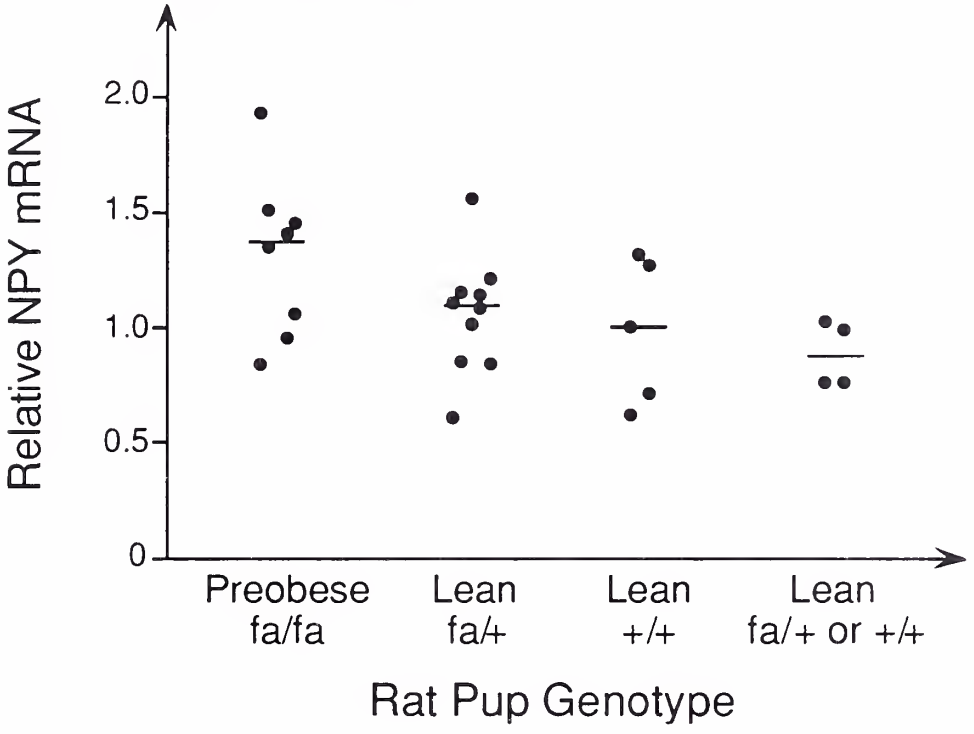
Assigned Genotype	Phenotype	# of Animals	NPY/Actin* mRNA median and range	Medians: preobese vs. lean
fa/fa	Preobese	8	1.37 (0.84-1.92)	1.37
fa/+	Lean	10	1.09 (0.61-1.56)	
+/+	Lean	5	1.00 (0.62-1.31)	1.00
fa/+ or +/+	Lean	4	0.88 (0.76-1.02)	

* NPY mRNA levels normalized to actin mRNA of the same sample. NPY/Actin levels are normalized to the median of the lean group

fa=13M allele
+=BN allele

The results show a statistically significant, $p<.021$, increase in NPY mRNA of 37% in the preobese group (median 1.37) when compared to the lean group (median 1.00). Further statistical analysis suggests that this effect may only be significant when lean homozygote (+/+) animals are considered in relation to their preobese counterparts. A comparison of the 5 +/+ animals to the 8 *fa/fa* animals shows a statistically significant elevation of NPY in the obese group, $p<.040$, the median of the *fa/fa* group is 1.40 times that of the +/+ group. Comparison of 10 heterozygote (*fa*/+) lean animals to the 8 preobese (*fa/fa*) animals shows a median of 1.00 for the lean group and 1.30 for the preobese group, but this difference is not statistically significant ($p >.050$).

Figure 9: Northern Blot Determination of Hypothalamic NPY mRNA Levels for 7-8 Day Old Lean and Preobese F2 Rat Pups of the 13MxBN Cross: Elevation in the Preobese Group*



* Rat pups were genotyped using rat chromosome 5 SSR polymorphisms for *Glut-1* and *C-Jun* in PCR linkage assays to the *fa* locus. Single NPY mRNA determinations were performed on each hypothalamic sample using the Northern blot technique. NPY mRNA levels were normalized to actin mRNA of the same sample. NPY/Actin levels were expressed by normalization to the median of the lean group. Bars represent the medians of each group. The results show a statistically significant, $p<.021$, increase in NPY mRNA of 37% in the preobese group (median 1.37) when compared to the lean group (median 1.00). Further statistical analysis suggests that this effect may only be significant when lean homozygote (+/+) animals are considered in relation to their preobese counterparts. A comparison of the 5 +/+ animals to the 8 *fa/fa* animals shows a statistically significant elevation of NPY in the obese group, $p<.040$, the median of the *fa/fa* group is 1.40 times that of the +/+ group. Comparison of 10 heterozygote (*fa*/+) lean animals to the 8 preobese *(fa/fa)* animals shows a median of 1.00 for the lean group and 1.30 for the preobese group, but this difference is not statistically significant ($p>.050$).

Six *fa/fa* and 9 lean [*fa/+* or +/+] animals were selected for RT-PCR analysis. A portion of the hypothalamic mRNA sample from these animals was reverse transcribed and assayed for NPY and actin mRNA by the PCR as outlined in the Materials and Methods section. Single measures were performed on each sample. The results indicate that the medians are virtually identical, 1.00 for the entire lean group and 1.04 for the obese group, and that there is no statistically significant difference between the two groups, ($p > .05$). However, the values obtained by the Northern blot method for these same samples show that the median of the obese group is 1.41, while that of the lean group is only 1.00. Further, the increased expression of NPY in the obese group, shown by the Northern blot method, is statistically significant, ($p < .025$). (See Table 11 and Figures 10 & 11).

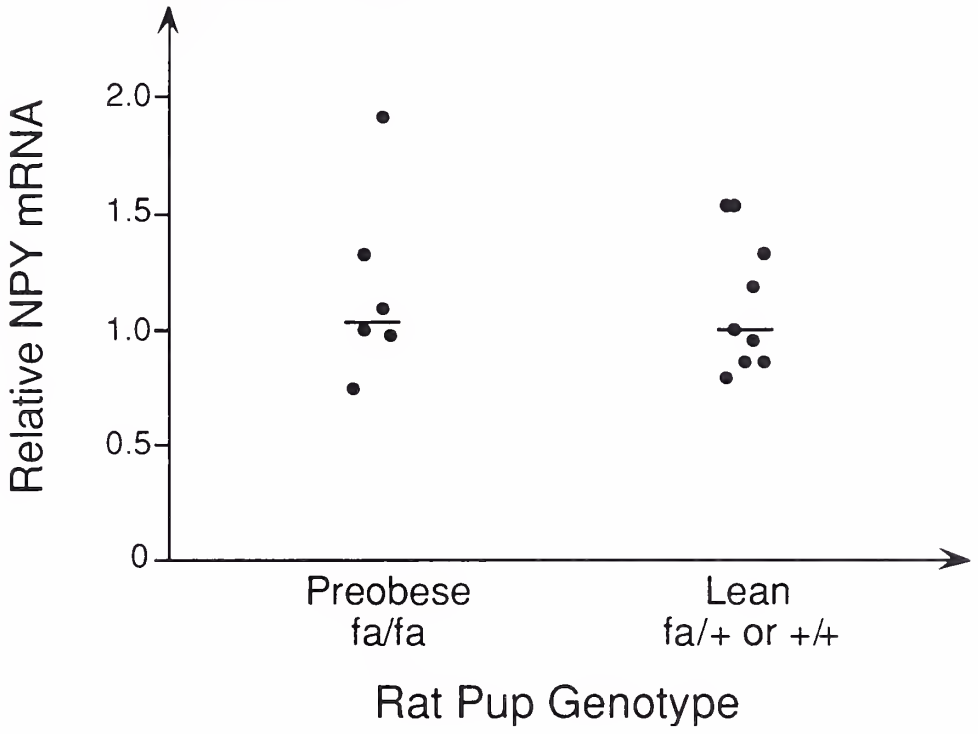
Table 11: Hypothalamic NPY mRNA levels for 7-8 day old F2 lean and preobese rat pups of the 13MxBN cross: RT-PCR shows no difference, while Northern blot shows elevation in the preobese group

Assigned Genotype	Phenotype	# of Animals	NPY/Actin* Median & Range by RT-PCR	NPY/Actin Median & Range by Northern Blot
fa/fa	preobese	6	1.04 (0.75-1.91)	1.41 (0.84-1.94)
+/+	lean	5	0.96 (0.79-1.53)	1.01 (0.63-1.32)
fa/+ or +/+	lean	4	1.16 (0.86-1.53)	0.89 (0.77-1.03)

* NPY mRNA levels normalized to actin mRNA of the same sample, and these values are then normalized to the median of the lean group for each method.

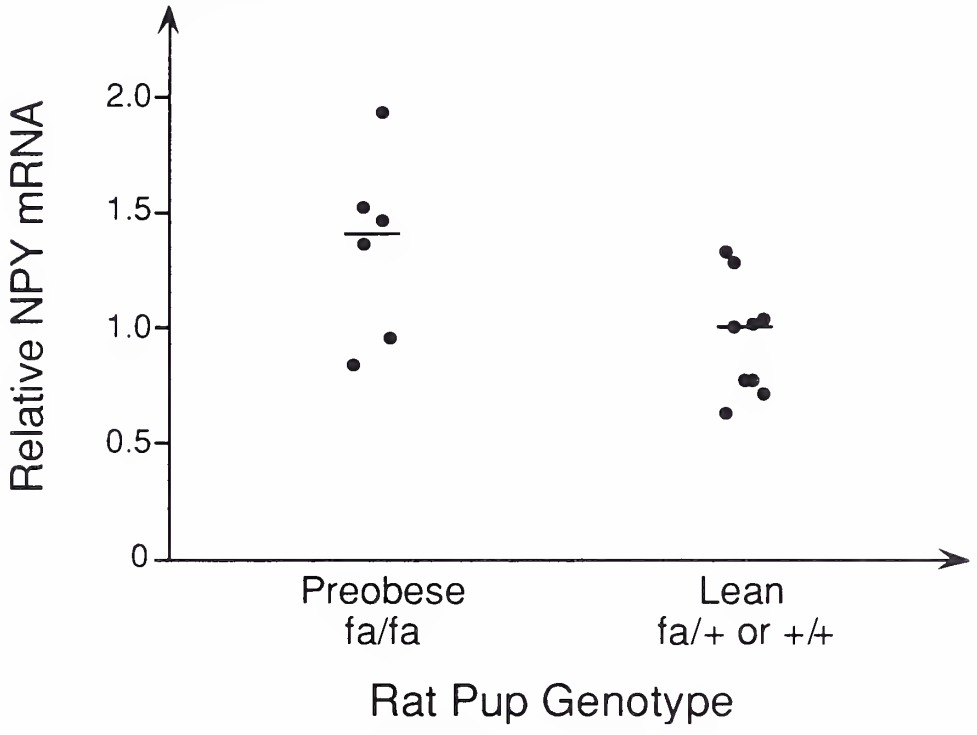
The medians for the RT-PCR method are virtually identical, 1.00 for the entire lean group and 1.04 for the obese group, and there is no statistically significant difference between the two groups, $p>.05$. However, the values obtained by the Northern blot method show that the median of the obese group is 1.41, while that of the lean group is only 1.00, and this difference is statistically significant, $p<.025$.

Figure 10: RT-PCR Determination of Hypothalamic mRNA Levels of 7-8 Day Old Lean and Preobese F2 Rat Pups of the 13MxBN Cross: No Difference Between Preobese and Lean Groups*



* Rat pups were genotyped using rat chromosome 5 SSR polymorphisms for *Glut-1* and *C-Jun* in PCR linkage assays to the *fa* locus. Single NPY mRNA determinations were performed on each hypothalamic sample using the RT-PCR technique. NPY mRNA levels are normalized to actin mRNA of the same sample. NPY/actin levels are expressed by normalization to the median of the lean group. Bars represent medians for each group. The medians are virtually identical, 1.00 for the entire lean group and 1.04 for the obese group, and there is no statistically significant difference between the two groups, $p>.05$.

Figure 11: Northern Blot Determination of Hypothalamic mRNA Levels of 7-8 Day Old Lean and Preobese F2 Rat Pups of the 13MxBN Cross: Elevation in the Preobese Group*



* Rat pups were genotyped using rat chromosome 5 SSR polymorphisms for *Glut-1* and *C-Jun* in PCR linkage assays to the *fa* locus. Single NPY mRNA determinations were performed on each hypothalamic sample using the Northern blot technique. NPY mRNA levels were normalized to actin mRNA of the same sample. NPY/actin levels were expressed by normalization to the median of the lean group. Bars represent medians for each group. Results show that the median of the obese group is 1.41, while that of the lean group is only 1.00, and this difference is statistically significant, $p<.025$.

Discussion

Hypothalamic NPY Elevation in Obese Zuckers by Age:

It has been demonstrated by several techniques that obese adult Zucker rats, of various ages, have elevated hypothalamic NPY levels. For example, a radioimmunoassay (RIA) technique has shown increased hypothalamic NPY peptide levels in 6-month-old obese Zucker rats in comparison to lean littermates.[3] A ribonuclease (RNase) protection assay has shown elevations of preproNPY (ppNPY) mRNA in obese Zucker rats at 15 and approximately 45 weeks of age, when compared to lean controls. These results were also confirmed using a Northern blot technique in a similar group of 45-week-old rats.[48]

Developmental studies have yielded conflicting results upon examination of younger Zucker rats. One study, using a RNase protection assay, demonstrated elevations of ppNPY mRNA in obese Zucker rats at 5, 14, and 33 weeks of age. Although elevations were found in these obese animals at each age, the difference found in the older rats was greater than that found in the 5-week-old group.[47] However, another developmental study, using the Northern blot technique, demonstrated increased NPY levels in obese *fa/fa* rats at 24 and 40 weeks of age, but no difference in expression between younger 11-week-old lean and obese rats.[29] At this same age of 11 weeks, another study, using an RT-PCR method, detected a 4-fold increase in NPY mRNA in the obese Zucker rat group.[2] The results of the present research also demonstrate an

approximate 50% increase in hypothalamic NPY mRNA, using a RT-PCR technique, in 11-week-old obese F2 13M/13M animals from the BNx13M cross. The discrepancies between these developmental studies may be partially accounted for by variations in hypothalamic NPY levels in younger rats, and by differences in NPY expression between the Zucker rat strain and rats of the BNx13M cross.

To determine the importance of elevated hypothalamic NPY in the production of the obese phenotype, the developmental analysis must be extended to the first week of life, an age at which other confounding phenotypic abnormalities of obesity are not yet present. A preliminary study using a RT-PCR technique failed to demonstrate differences in hypothalamic NPY mRNA levels between lean and preobese 13-day-old Zucker rat pups.[2] On the other hand, a study of six-to-nine day old rat pups of the BNx13M cross, using a RNase protection assay technique, demonstrated a 50% elevation of hypothalamic ppNPY in the preobese group when compared to heterozygous lean animals.[16] The present research also yields differing results, depending on the technique used. The newer RT-PCR method detects no difference between lean and preobese rat pups aged 6-to-8 days old. However, the Northern blot technique demonstrates a statistically significant 37% elevation of hypothalamic NPY levels in preobese 7-to-8 day old pups.

Acceptance of Northern Blot Results:

A thorough consideration of the two techniques used in this research suggests that more confidence may be placed in the results obtained by the Northern blot. First, the Northern blot technique has been extensively used to determine NPY mRNA in adult rodents,[15,29,48] and the results obtained have been reproducible by other techniques, including radioimmunoassay and RNase protection assay.[3,4,9,46-48] Only one preliminary study using an RT-PCR technique to detect NPY mRNA has been performed,[2] and the results are not entirely consistent with results obtained in similar studies using other techniques.[16,29,47] Second, the results obtained using the Northern blot method in this study are consistent with those found in a recent study using another standard technique, the RNase protection assay.[16] Only RT-PCR methods, used to detect NPY mRNA in this research, and in a preliminary report by another laboratory,[2] have failed to detect any differences in NPY expression between lean and preobese pups. Third, there is more possibility for error in measurements performed with amplification of target molecules. The RT-PCR method amplifies the signal for each target RNA molecule between 10^4-10^6 fold, whereas the Northern blot and RNAase protection assays determine RNA levels on a molar basis (one molecule of probe detects one molecule of target RNA). Lastly, the differences in NPY expression between the two groups are relatively small, and it may be

that the RT-PCR method is not sufficiently sensitive to reliably detect variations of 50% or less.

The inability of the RT-PCR method to detect small differences in product amount may be secondary to determining a visual, rather than a mathematical standardization curve[13] for the oligonucleotide primer amplifications. To establish a standardization curve for this research, sequential PCR amplifications were performed, and the cycle number (ie. 20) at which the NPY and actin products appeared to be still increasingly linearly, before reaching a plateau phase, was determined and subsequently used. Because of differential primer amplification, this visual standardization curve may not have been accurate enough to identify relatively small, but significant differences in NPY expression. Furthermore, actin may not have been the most appropriate control product to generate, because it is present in much greater amounts than NPY. This fact necessitated the use of much longer autoradiogram exposure times to ascertain NPY levels (ie. typical exposure time for NPY was > 72 hours, while for actin it was < 24 hours). Even at very short exposure times, actin densitometry values may have already been within saturation range. In addition, on the longer exposures for NPY, high background levels of radiation may have yielded inaccurate NPY densitometry values.

The above arguments support the validity of the Northern blot results, which demonstrate a 37% elevation of NPY in the preobese rat pups. Similar results obtained by a RNase

protection assay study demonstrate a greater degree of NPY elevation in the preobese group, of 50%.[16] This slight discrepancy may be accounted for by the use of only homozygous lean animals in that study,[16] whereas both homozygous and heterozygous lean animals were used in the Northern blot study. It is possible that there is an allelic effect of the *fatty* mutation such that heterozygous animals have intermediate levels of hypothalamic NPY. Of additional note, the demonstrated degree of elevation in the preobese pups in both of these studies is less than that observed in older obese rats.[3,29,47,48] One possible explanation for this finding may be that although the pups within a litter are of the same gestational age, the pups may differ in developmental maturity, such that the margin of difference in hypothalamic NPY expression between lean and preobese pups may be narrower than that found later in life.

Strain Considerations:

The ramifications of using the 13MxBN cross, which is not genetically identical to the Zucker rat strain, should be considered. The 13M allele from the Zucker rat strain was introduced into the BN rat strain. Thus, variability in NPY expression may be secondary to differences between the 13M and BN strains, rather than due to the *fa* mutation itself. To ascertain whether early hypothalamic NPY expression is influenced by strain differences in rodent obesity models, it will be necessary to evaluate hypothalamic NPY expression in

a single strain model, (ie., a Kaliss mouse strain: C57BL/KsJ), which can be phenotyped at a very early age.

Another implication of using two strain backgrounds is genetic dissimilarity between *fa/fa* animals. All progeny of 13MxBN F1 animals have Mendelian segregation of 13M and BN alleles at all loci not linked to the *fa* gene. In addition, recombinations may occur, such that in any given *fa/fa* animal, 5-10% of its genes may be heterozygous. Therefore, variability in the expression of the obese phenotype will occur if it is influenced by other polygenes, which is likely, since the *fa/fa* rats are not genetically identical (13M/13M) at other loci in the genome.

Possible Role of NPY in the Development of Obesity:

The Northern blot demonstration of the early onset of hypothalamic NPY elevation in preobese rat pups is significant because centrally acting NPY can produce metabolic aberrations which likely play a central role in the development of the obese *fatty* phenotype. One significant consequence of central NPY action is pancreatic release of the anabolic hormone insulin,[39] which facilitates deposition of glucose as adipose tissue. Hyperinsulinemia is evident in the Zucker rats by 3 weeks of age;[58] in the *diabetes* mouse, the homologue of the *fatty* rat, hyperinsulinemia has been detected as early as 10 days of age.[18] Although increased hypothalamic NPY expression is evident prior to the onset of hyperinsulinemia, the earliest known defect in insulin

secretion in preobese Zucker rats is enhanced *in vitro* secretion in response to acetylcholine perfusion, at 5 days of age.[1] One possible theory regarding the mechanism of obesity in *db* mice,[18] which can be generalized to *fatty* rats, suggests that hyperinsulinemia in preobese rodent pups causes a mild hypoglycemia, followed by a compensatory hyperphagia to return blood glucose levels to near normal, which in turn results in enhanced insulin secretion and fat deposition.[18] It may be that heightened hypothalamic NPY expression initiates this entire process culminating in obesity.

Another metabolic consequence of centrally acting NPY is improved energy efficiency. Intracerebroventricular NPY administration in rats produces a hypometabolic condition by eliciting decreases in heart rate, respiratory rate, and mean arterial blood pressure. However, these effects do not occur if food is concomitantly consumed, presumably because addition of an energy source obviates the need for conserving energy expenditure.[26] NPY also lowers body temperature,[30] leading to an overall state of decreased activity resembling hibernation. Not surprisingly, conditions in which energy conservation is required for survival, such as fasting and cold exposure, have been shown to elicit elevations of hypothalamic NPY, presumably initiating these energy conserving mechanisms.[15,34] Given the increased hypothalamic NPY levels of *fa/fa* rats, these same mechanisms presumably operate, likely facilitating the increased energy storage in these animals.

Hypothalamic NPY in Humans:

Central NPY has been assayed in the cerebrospinal fluid (CSF) of human beings with eating disorders.[32] In anorexia nervosa patients, NPY levels were found to be elevated during periods of starvation, and were normalized after long-term weight restoration accompanied by regular eating habits.[32] These results are consistent with the relative NPY elevations found in rodents during periods of food deprivation, in comparison to those obtained after refeeding.[4,9,15,29,46,48] Bulimic patients also had significantly increased amounts of peptide YY (closely related to NPY) during episodes of abstinence from bingeing, compared to their levels during actively bingeing periods.[32] These results suggest that bulimics have abnormally high levels of peptide YY which may be the stimulus for them to engage in periods of gross hyperphagia. Although hypothalamic NPY cannot be assayed directly in human beings, the above findings suggest that determination of CSF levels may be an accurate reflection of hypothalamic NPY production, and that CSF NPY may be elevated in obese patients. Thus, studies assessing central NPY levels in obese humans should be performed to determine if the NPY findings in obese animal models are relevant to human beings.

NPY Antagonists:

Given that excess hypothalamic NPY can produce obesity,[50] it is important to identify any agent capable of counteracting the effects of this potent neurotransmitter. The serotonergic drug fenfluramine is a clinically prescribed anorectic which has been shown to cause rapid and dramatic decreases in hypothalamic NPY levels in rats treated with the drug, suggesting that fenfluramine mediates its appetite-suppressant effects by inhibition of hypothalamic NPY activity.[45] However, fenfluramine is characterized by significant side effects, and appears unable to sustain long-term weight reduction.[25]

Recently the cobalt substituted heme compound, cobalt-protoporphyrin (CoPP), was shown to dramatically reduce both food intake, and weight in rats (including obese *fatty* rats), mice, chickens, and dogs when injected either peripherally, intracerebroventricularly,[23] or directly into the hypothalamus.[22] CoPP causes an initial 2-3 day period of hypophagia coupled with marked weight reduction, followed by resumption of normal food intake levels, and a gradual return to normal body weight 200-300 days after CoPP injection. This dramatic weight loss persists long after food intake has returned to normal, suggesting a CoPP-mediated aberration of body fat metabolism.[23]

Investigations suggest that CoPP produces weight loss by interfering with hypothalamic NPY action. Hypophagic CoPP-treated rats were shown to have an appropriate increase in

hypothalamic NPY mRNA, but this was not translated into the expected corollary of food ingestion.[22] Intracerebroventricular NPY administration to CoPP-treated rats also did not increase food intake as anticipated.[22] Since CoPP did not block the production of hypothalamic NPY, and exogenous NPY did not induce feeding, additional studies have focused on the first site of NPY action, its hypothalamic receptor. These studies, performed in rats, have demonstrated that CoPP treatment does not alter either the number of NPY receptors, or their affinity for NPY. Thus, the mechanism of action of CoPP must be distal to the NPY receptor(Michael Turner-personal communication). Further explorations into CoPP, and other antagonists[38,55] of the orexigenic effect of NPY may eventually lead to a viable pharmacologic agent for the treatment of hypothalamic NPY-mediated obesity.

Conclusion:

Human obesity is considered to have both behavioral and genetic components. The Zucker rat provides a valuable model of an inherited obesity syndrome caused by an unidentified autosomal recessive mutation of the *fatty (fa)* gene on chromosome 5. The development of obesity in animal models appears to be influenced by hypothalamic neuropeptide Y (NPY), via metabolic aberrations inducing hyperphagia, hyperinsulinemia, and energy conservation mechanisms. Elevated hypothalamic NPY levels have been consistently

demonstrated in obese adult Zucker rats in comparison to lean Zucker rats.[3,29,47,48] However, until recently, it has not been possible to assess the causal role of NPY in the development of this obesity, because the genotype of Zucker rat pups could not be determined prior to the onset of visible obesity. The creation of a new rat cross (13MxBN), similar to the Zucker rat strain (13M/13M), and identification of polymorphic genetic markers flanking the *fa* locus has permitted genotyping of 13MxBN rats at any age. The purpose of the present study was to elucidate the potential role of hypothalamic NPY in the development of the obese phenotype of the *fatty* rat, by comparing hypothalamic NPY levels in lean and preobese 13MxBN rat pups, using the RT-PCR and Northern blot methods. The RT-PCR technique, which requires amplification of target sequence, showed no statistically significant difference between 6-8 day-old lean and preobese pups. However, the Northern blot method, which detects native mRNA, demonstrated a significant elevation of ~40%, ($p<.021$), in preobese rat pups at 7-8 days of age, compared to their lean littermates. Furthermore, the Northern blot results are consistent with those recently obtained with an RNAase protection assay.[16] These studies provide evidence that elevated hypothalamic NPY is one of the earliest known molecular abnormalities in a genetic animal model of obesity, and suggest that NPY is integrally involved in producing the obese phenotype. Studies of central NPY expression in human beings with eating disorders suggest that

the NPY elevations in obese rodents may be relevant to obese humans, and imply that investigations into hypothalamic NPY antagonists may eventually lead to a pharmacolgic agent for the treatment of obesity.

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